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MONOCLONAL ANTIBODIES AGAINST VERO CELLS THAT PROTECT AGAINST DIPHTHERIA TOXIN

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B. J. RÖNNBERG, B. C. LIDGERDING and J. L. MIDDLEBROOK. Monoclonal antibodies against Vero cells that protect against diphtheria toxin. *Toxicon* 27, 1095-1104, 1989.—Mice were immunized with a cell line (Vero) that possesses a high number of membrane receptors for diphtheria toxin. Spleen cells from these mice were fused with SP2/0-Ag14 cells and two cell lines (1A2 and 2D2) isolated by screening for the ability of their secreted antibodies to inhibit binding of radiolabeled diphtheria toxin to Vero cells. These antibodies protected Vero cells from the inhibition of protein synthesis mediated by diphtheria toxin. The antibodies were purified, iodinated, and their binding characteristics investigated. At 4°C, the association of 1A2 and 2D2 with Vero cells was saturable ($K_D \approx 10^{-8}$ M) and indicated about 10^6 binding sites/cell. Diphtheria toxin did not inhibit the binding of either radiolabeled antibody. Monoclonal antibody 1A2 completely inhibited ¹²⁵I-2D2 binding and vice versa. Trypsin or phospholipase C treatment of Vero cells had no effect on the ability of the monoclonal antibodies to bind to the cells. These findings suggest that: (1) the two monoclonal antibodies recognize the same or closely related epitopes and (2) the antibodies bind a domain distinct from the toxin binding site or to a subcomponent of the diphtheria toxin receptor that is present at many other cell surface sites. These antibodies offer a powerful tool to study the structure, processing and mode of action of diphtheria toxin receptors.

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INTRODUCTION

DIPHTHERIA toxin is produced by *Corynebacterium diphtheriae* lysogenic for phage carrying the *tox* gene. The toxin is secreted as a single polypeptide chain (M_r 58,342) with two disulfide bonds and no free sulfhydryl groups. Limited proteolysis yields an amino terminal fragment A (M_r 21,167) and a carboxy terminal fragment B (M_r 37,195) which remain associated via a disulfide bridge (PAPPENHEIMER and GILL, 1973; COLLIER, 1975). Intoxication of susceptible cells by diphtheria toxin involves binding of the toxin, through fragment B, to specific cell-membrane receptors, followed by translocation of the enzymatically active fragment A into the cytoplasm. Fragment A then catalyzes transfer of ADP-ribose from NAD⁺ to elongation factor 2, resulting in an inactive elongation factor 2 and the arrest of protein synthesis (PAPPENHEIMER and GILL, 1973; COLLIER, 1975).

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It is a widely held belief that diphtheria toxin is internalized by receptor-mediated endocytosis. This process involves binding of the toxin to specific receptors on the target cell; clustering of the toxin-receptor complexes over specialized, clathrin-coated regions of the membrane; and internalization in endosomes (EIDELS *et al.*, 1983; MIDDLEBROOK and DORLAND, 1984). The intracellular compartment, where endocytosed diphtheria toxin encounters a low pH that initiates penetration of fragment A through a membrane into the cytoplasm, has been suggested to be a prelysosomal vesicle (EIDELS *et al.*, 1983; MIDDLEBROOK and DORLAND, 1984).

Although receptor-mediated endocytosis and receptor recycling have been extensively documented for hormone and growth factor receptors (BROWN *et al.*, 1983; WILEMAN *et al.*, 1985; DAUTRY-VARSAT, 1986), little is known about the structure, biosynthesis and possible recycling of bacterial toxin receptors. Efforts to study the biosynthetic regulation of diphtheria toxin receptors have been hampered due to the lack of antibodies against the receptor. In the present study, mice were immunized with intact Vero cells, a cell line with a large number of cell surface diphtheria toxin receptors (MIDDLEBROOK *et al.*, 1978). Spleen cells of mice immunized by this protocol were fused with SP2/0-Ag14 cells and two hybridomas were identified that produced antibodies inhibiting the binding of radiolabeled toxin to Vero cells. The characteristics of these monoclonal antibodies are the subject of this report.

MATERIALS AND METHODS

Materials

Diphtheria toxin was obtained from Connaught Laboratories and purified by chromatography over DE52 (Whatman) (MIDDLEBROOK *et al.*, 1978). ^{125}I -labeled toxin was prepared by the chloramine-T method, as previously described (MIDDLEBROOK *et al.*, 1978), to a specific activity of $1-2 \times 10^7$ cpm/ μg of toxin. 'Low pH' carrier-free Na^{125}I and L-[4,5- ^3H]-leucine (120 Ci/mmol) were obtained from Amersham. Trypsin and phospholipase C were purchased from Sigma. Polyethylene glycol 1500 was obtained from Boehringer Mannheim. Other chemicals used were of reagent grade.

Cells

Seed stocks for MRC-5 cells were obtained from the Salk Institute, Vero cells from the Centers for Disease Control, and LLC-MK2, CHO and L-929 cells from the American Type Culture Collection (ATCC). Cells were maintained in 75 cm² T-flasks (Costar No. 3075) or 700 cm² roller bottles (Costar No. 1234) with the medium and serum supplement recommended by ATCC. The non-immunoglobulin-secreting murine plasmacytoma SP2/0-Ag14 cell line (SHULMAN *et al.*, 1978) was grown in Optimem medium (Gibco) with 7.5% fetal bovine serum (Armour Pharmaceutical), 2 mM glutamine, 50 $\mu\text{g}/\text{ml}$ gentamicin (Whittaker Bioproducts), 2 $\mu\text{g}/\text{ml}$ Fungizone (Gibco) and 15% conditioned medium. Conditioned medium was prepared by filtration of Eagle's minimum essential medium from 7-14 day old MRC-5 cells through a 0.01 μm filter (Millipore).

Immunization procedure

Female Balb/c mice were injected i.p. with 10^7 EDTA-released (1 mM EDTA in Hanks' balanced salt solution without Ca^{2+} and Mg^{2+}) Vero cells three times at 3 week intervals. Four days after the last i.p. injection, the mice were sacrificed for the fusion experiment.

Fusion and selection of hybridomas

Spleen cells from the immunized mice were fused with SP2/0-Ag14 cells at a 2.5:1 ratio, using 50% (v/v) polyethylene glycol 1500 as fusogen, following the procedure of EARLY and OSTERLING (1985). Hybrid cells growing in Optimem medium with hypoxanthine, aminopterin and thymidine, were assayed for secretion of mouse Ig by an ELISA using affinity-purified, peroxidase-labeled, goat anti-mouse Ig antibodies (Hyclone). Hybrid cultures secreting Ig and positive in the screening assay described below were cloned by limiting dilution on macrophage feeder layers (RENER *et al.*, 1985). Cells from isolated clones were grown in culture and in Balb/c strain CJ mice as ascitic fluid. The isotype and sub-isotype of the produced monoclonal Ig were determined by an ELISA using monospecific antisera (Hyclone). Monoclonal antibodies (IgG₁) were isolated from ascites fluid by

Monoclonal antibody screening assay

Diphtheria toxin binding

Monoclonal antibody binding

Assay for macromolecular synthesis

RESULTS

Mice were immunized i.p. with 10^7 Vero cells that had been removed from the plastic surface of the 75 cm² T-flasks by treatment with EDTA (1 mM EDTA in Hanks' balanced salt solution without Ca²⁺ and Mg²⁺). Scraping cells off the plastic surface with a cell scraper almost completely abolished their ability to bind toxin (data not shown). One week after the second immunization, the antisera of two mice were able to inhibit ¹²⁵I-diphtheria toxin binding to Vero cells (data not shown). Four days after the third immunization, spleen cells of these two mice were fused with SP2/0-Ag14 cells. We first selected hybrid cultures that secreted mouse Ig by screening their media with an ELISA. The ability of the secreted Ig to inhibit ¹²⁵I-diphtheria toxin binding to Vero cells was then screened by a binding assay. The hybrid cells from two positive cultures were cloned by limiting dilution. Two fast-growing clones that secreted IgG₁ (denoted 1A2 and 2D2) were grown in large quantities as ascites in mice and the IgG₁ purified on protein A-agarose. The inhibition of ¹²⁵I-diphtheria toxin binding to Vero cells by the monoclonal antibodies is shown in Fig. 1. Inhibition of toxin binding was observed at antibody concentrations as low as 5–10 nM, while maximal binding inhibition was obtained at 50 nM.



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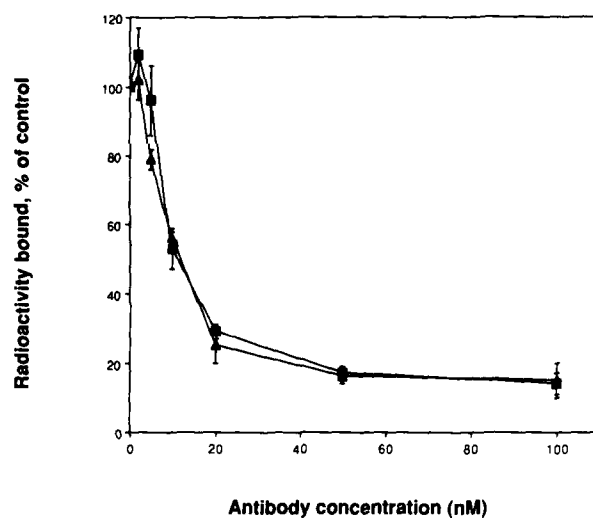


FIG. 1. INHIBITION OF ^{125}I -DIPHThERIA TOXIN BINDING TO VERO CELLS BY AFFINITY-PURIFIED MONOCLONAL ANTIBODIES.

Inhibition of binding of 0.5 nM ^{125}I -diphtheria toxin to Vero cells by increasing concentrations of monoclonal antibodies. Cells were incubated with a mixture of toxin and antibodies for 24 hr at 4°C. Triplicate samples were processed and counted as described under Materials and Methods. 1A2 (■) and 2D2 (▲). Error bars indicate S.E.M.

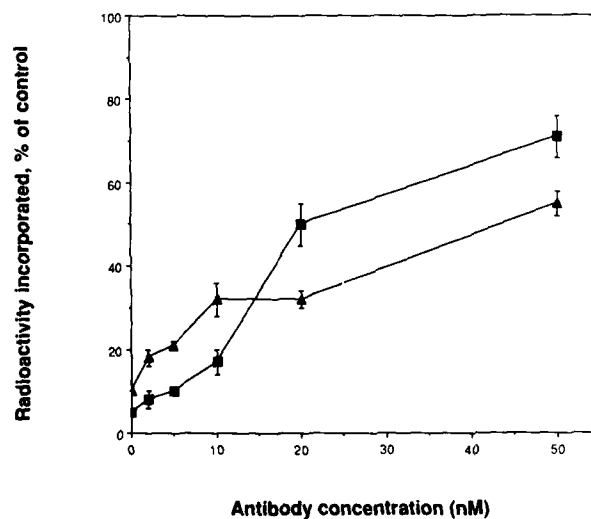


FIG. 2. PROTECTIVE PROPERTIES OF THE MONOCLONAL ANTIBODY 1A2.

Cells were incubated for 24 hr at 4°C with 0.5 nM diphtheria toxin + monoclonal antibody 1A2 in the concentration range 0–50 nM, then transferred to 37°C and incubated for an additional 2 hr. Tritiated leucine was added during the last 0.5 hr at 37°C. Triplicate samples were processed and counted as described under Materials and Methods. Control leucine incorporation was measured without toxin or antibody. Error bars indicate S.E.M. Vero cells (■) and LLC-MK2 cells (▲).

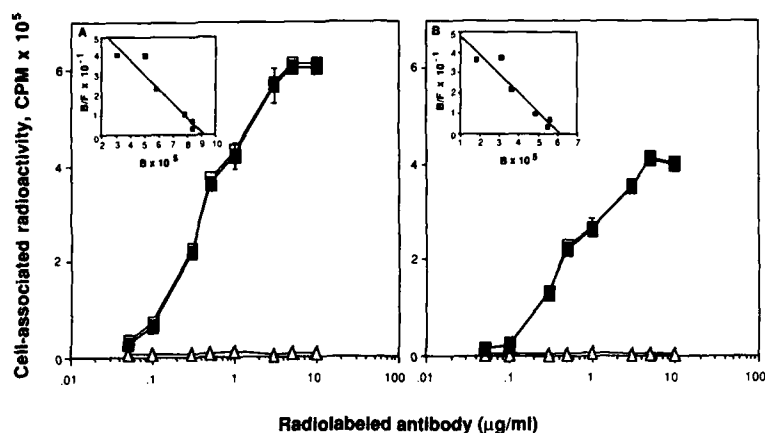


FIG. 3. EFFECT OF RADIOLABELED MONOCLONAL ANTIBODY CONCENTRATION ON ANTIBODY-CELL ASSOCIATION.

Vero cells were incubated for 24 hr at 4°C with ^{125}I -monoclonal antibodies in the concentration range 0.05–10 $\mu\text{g/ml}$. Nonspecific binding was determined in the presence of a 100-fold excess of unlabeled antibodies. Antibody-cell binding (in triplicate) was measured as described under Materials and Methods and the data treated by the method of SCATCHARD (1949). (A) ^{125}I -1A2 (\square), ^{125}I -1A2 + unlabeled 1A2 (\triangle), difference between (\square) and (\triangle) (\blacksquare). (B) ^{125}I -2D2 (\square), ^{125}I -2D2 + unlabeled 2D2 (\triangle), difference between (\square) and (\triangle) (\blacksquare). Error bars indicate S.E.M., which, when not shown, are smaller than the symbols.

The ability of the monoclonal antibodies to protect cells from diphtheria toxin was determined. Besides the Vero cell line, we challenged another highly toxin-sensitive cell line, LLC-MK2, and a moderately toxin-sensitive cell line, CHO. Figure 2 shows that 1A2 protected both Vero and LLC-MK2 cells in a dose-related manner from diphtheria toxin-induced inhibition of protein synthesis. In contrast, there was no detectable protection of CHO cells at antibody concentrations up to 100 nM. Similar results were obtained with antibody 2D2 (data not shown).

The purified monoclonal antibodies were iodinated by the same technique as for diphtheria toxin. Specific activities of $4\text{--}6 \times 10^6 \text{ cpm}/\mu\text{g}$ were usually obtained. Binding isotherms for the association of these two monoclonal antibodies with Vero cells were carried out with the results shown in Fig. 3. The association was highly specific, as judged by competition with a 100-fold excess of unlabeled homologous antibody. Specific association increased as a function of labeled antibody concentration up to about 5 $\mu\text{g/ml}$. The inset in Fig. 3 shows a re-plot of the specific association data by the method of SCATCHARD (1949). The results of this data transformation were consistent with a single class of binding sites ($1.5 \times 10^6/\text{cell}$; range $0.62\text{--}2.4 \times 10^6/\text{cell}$) with an apparent dissociation constant of $0.66 \times 10^{-8} \text{ M}$ (range $0.29\text{--}1.1 \times 10^{-8} \text{ M}$) for both antibodies.

The kinetics of labeled antibody association with Vero cells are shown in Fig. 4. The kinetics exhibited a classical bimolecular reaction pattern at both 4 and 37°C. The rate of association was slower at 4 than 37°C, reaching a maximum after 24–32 hr compared to 4–6 hr at physiological temperature. The magnitude of specific association also reached a higher level at 37°C compared to that attained at 4°C. The specificity of association at both temperatures was high, more than 95% of the total.

Because their binding properties were so similar, we asked whether the two monoclonal antibodies would compete with each other for binding to Vero cells. Figure 5 shows that

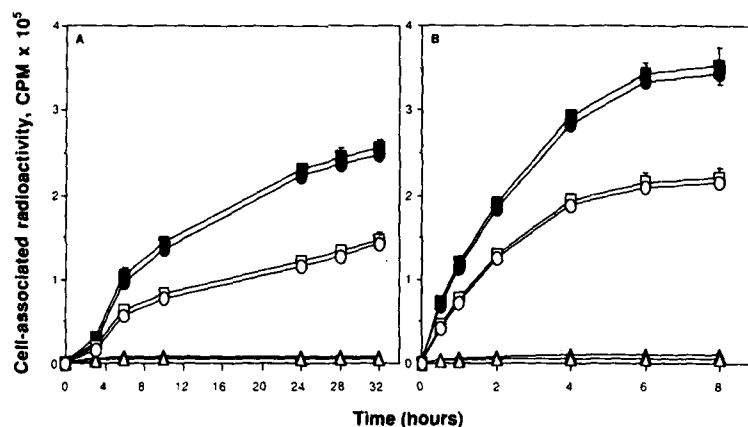


FIG. 4. KINETICS OF ^{125}I -LABELED MONOCLONAL ANTIBODY-VERO CELL ASSOCIATION. ^{125}I -monoclonal antibody (0.25 $\mu\text{g}/\text{ml}$) or ^{125}I -monoclonal antibody plus unlabeled antibody (25 $\mu\text{g}/\text{ml}$) was added to the cells. At the times indicated, triplicate samples were processed and counted as described under Materials and Methods. (A) 4°C and (B) 37°C. ^{125}I -1A2 (■), ^{125}I -1A2 + unlabeled 1A2 (▲), difference between (■) and (▲) (●), ^{125}I -2D2 (□), ^{125}I -2D2 + unlabeled 2D2 (△), and difference between (□) and (△) (○). Error bars indicate S.E.M. which, when not shown, are smaller than the symbols.

homologous and heterologous antibodies competed equally well for binding of the radiolabeled monoclonal IgG₁. In contrast with this inhibition pattern, a 100-fold molar excess unlabeled diphtheria toxin did not measurably influence the binding of either antibody to the target cells (Fig. 5).

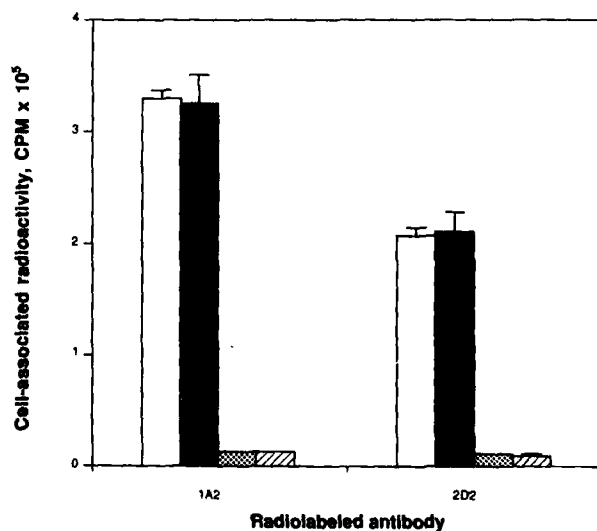


FIG. 5. COMPETITION OF MONOCLONAL ANTIBODY BINDING TO VERO CELLS. Cells were incubated with a mixture of radiolabeled antibodies (0.25 $\mu\text{g}/\text{ml}$) and a 100-fold molar excess of competitor for 24 hr at 4°C. Triplicate samples were processed and counted as described under Materials and Methods. Competitor added: none, open bars; diphtheria toxin, filled bars; 1A2, dotted bars; 2D2, hatched bars. Error bars indicate S.E.M., which, when not shown, are smaller than the line width.

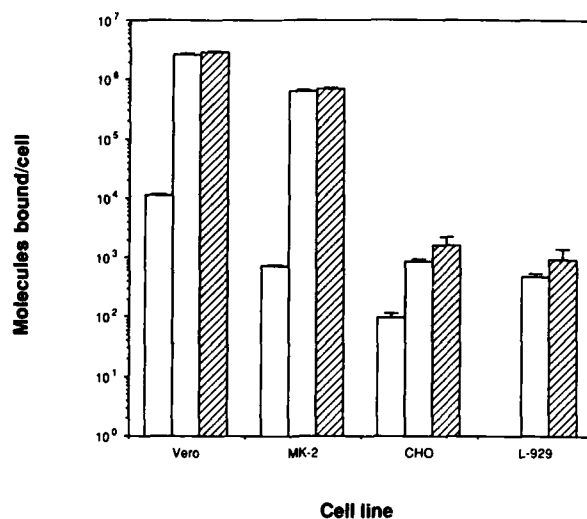


FIG. 6. BINDING OF RADIOLABELED DIPHTHERIA TOXIN AND MONOCLONAL ANTIBODIES TO VERO, LLC-MK2, CHO AND L-929 CELLS.

¹²⁵I-monoclonal antibody (0.25 μ g/ml), ¹²⁵I-monoclonal antibody plus unlabeled antibody (25 μ g/ml), ¹²⁵I-diphtheria toxin (0.03 μ g/ml), or ¹²⁵I-diphtheria toxin plus unlabeled toxin (3 μ g/ml) were added to the cells and incubated for 24 hr at 4°C. Triplicate samples were processed and counted as described under Materials and Methods. Diphtheria toxin (open bars), 1A2 (dotted bars), and 2D2 (hatched bars). Error bars indicate S.E.M. for the + direction.

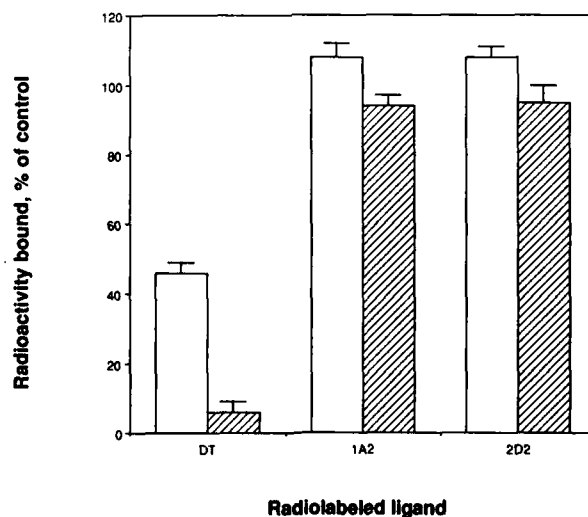


FIG. 7. BINDING OF RADIOLABELED DIPHTHERIA TOXIN AND MONOCLONAL ANTIBODIES TO TRYPSIN- OR PHOSPHOLIPASE C-TREATED VERO CELLS.

Cells in medium without serum were incubated with 10 μ g/ml trypsin for 60 min at 37°C or 5 μ g/ml phospholipase C for 30 min at 37°C, washed, then ¹²⁵I-monoclonal antibody (0.25 μ g/ml), ¹²⁵I-monoclonal antibody plus unlabeled antibody (25 μ g/ml), ¹²⁵I-diphtheria toxin (0.03 μ g/ml), or ¹²⁵I-diphtheria toxin plus unlabeled toxin (3 μ g/ml) were added to the cells and incubated for 24 hr at 4°C in complete H-199. Triplicate samples were processed and counted as described under Materials and Methods. Trypsin (dotted bars) and phospholipase C (hatched bars). Error bars indicate S.E.M.

Figure 6 shows the results of experiments comparing monoclonal antibody binding to the toxin-sensitive mammalian cell lines used for protection experiments, and one resistant to diphtheria toxin (L-929). Compared to Vero and LLC-MK2 cells, CHO cells bound almost 1000-fold fewer antibody molecules and 10–100-fold less diphtheria toxin. An even lower toxin and antibody binding to L-929 cells was observed. The number of toxin molecules bound to L-929 cells (and associated radioactivity) was so low that counts were not statistically different than 0. On the other hand, binding of antibodies to L-929 cells, while much lower than to Vero cells, was statistically real.

It has been shown that treatment of cells with trypsin or phospholipase C lowers their sensitivity to diphtheria toxin (MOEHRING and CRISPELL, 1974). More recent reports from this (RÖNNBERG and MIDDLEBROOK, submitted for publication) and another laboratory (OLSNES *et al.*, 1985) have indicated that the basis of protection is enzyme-catalyzed removal of the toxin receptor. When we examined the effects of these enzymes on monoclonal antibody binding, we obtained the results shown in Fig. 7. Neither enzyme reduced the binding of either monoclonal antibody to Vero cells, while trypsin reduced toxin binding about 50% and phospholipase C virtually eliminated diphtheria toxin binding.

DISCUSSION

We are interested in defining the biosynthesis, regulation and turnover of the receptor for diphtheria toxin. The receptor is present on the surface of target cells (DORLAND *et al.*, 1979) and appears to be the major factor determining whether or not a cell is susceptible to the toxin (MIDDLEBROOK *et al.*, 1978). In other systems where the biosynthesis of cell surface proteins or receptors was studied (VAN OBBERGHEN *et al.*, 1981; KIM *et al.*, 1987; WRIGHT *et al.*, 1987; KEATING and WILLIAMS, 1987; DEFIZE *et al.*, 1987), the availability of a receptor-specific antibody for immunoprecipitations was critical to success. We sought to obtain a diphtheria toxin receptor-specific monoclonal antibody by immunizing mice with Vero cells, a cell line that carries a large number of diphtheria toxin receptors on its surface (MIDDLEBROOK *et al.*, 1978). Vero cells are adherent, so it was necessary to remove them from the growth flask to inject into mice. Because trypsin or other protease treatment strips off the receptor (DORLAND *et al.*, 1979), we used a gentle method of removal, scraping the cells from the flask with a rubber policeman. While not harmful to the cells (as judged by replating), this technique consistently removed a substantial fraction of the receptors, up to 80%. We are unaware of previous reports where other toxin or hormone cell surface receptors are removed by such treatment, however, it is likely that such a possibility was not monitored. This phenomenon is probably an important clue to the structure or membrane-anchoring of the diphtheria toxin receptor, although its physiochemical basis is presently unclear.

By the use of EDTA, diphtheria toxin receptor bearing cells were obtained and, via standard hybridoma techniques, two IgG₁-secreting cell lines were obtained which blocked the binding of toxin to its receptor. The properties of these two monoclonal antibodies are indistinguishable, so it is quite possible that the two cell lines were derived from the same lymphocytic clone. In any event, the binding of the antibodies to Vero cells was very tight, with a K_D of approximately 10^{-8} M. The specificity of binding, as judged by competition with unlabeled monoclonal antibody, was high. However, several properties of the antibody binding sites on Vero cells did not correlate with properties of the toxin binding sites. First, at saturating concentrations, about 100-fold more antibody molecules bound

to the Vero cells than did toxin molecules. Second, the binding of antibody was not sensitive to protease or phospholipase C treatment of the cells, whereas toxin binding was much reduced by either enzyme treatment. On the other hand, there was a correlation between cell line sensitivity to the toxin and the level of both toxin and antibody binding. Also, the antibodies blocked the binding of diphtheria toxin and protected cells from the toxin, both with Vero cells and another sensitive cell line, LLC-MK2. Antibody-mediated protection from toxin did not occur with CHO cells, a cell line about 1000-fold less sensitive to diphtheria toxin than Vero or LLC-MK2. Because we did observe antibody binding to CHO cells, although much lower than to Vero cells, it is possible that the toxin receptor on CHO cells is different than the receptor on the more sensitive cell lines.

Several other laboratories have asked whether resistant cells carry a receptor for diphtheria toxin. This is not a trivial question since it is clear that protein ligands can bind very specifically to sites that are not receptors (CUATRECASAS and HOLLENBERG, 1975). CHANG and NEVILLE (1978) studied the binding of diphtheria toxin to plasma membranes from L-929 cells. They described toxin binding parameters which were markedly different from toxin binding to the receptor on Vero cells (MIDDLEBROOK *et al.*, 1978). Thus the question remains as to whether they defined toxin binding sites or toxin receptors. KEEN *et al.* (1982) examined the interaction of fluorescently-labeled diphtheria toxin with 3T3 cells and observed binding. However, since 3T3 cells are resistant to diphtheria toxin (MIDDLEBROOK and DORLAND, 1977) they were unable to distinguish between binding sites or receptors that mediate toxicity. More recently, MEKADA *et al.* (1988) found two diphtheria toxin-interactive substances in membrane preparations from Vero cells. One was a protein and may be a component of the receptor. The other was a nonprotein 'inhibitor' and was obtained in equal amounts from Vero and L-929 cell membranes. This 'inhibitor' could represent a candidate antigen against which our monoclonal antibodies are directed. However two observations mitigate against that possibility. First, Vero cells bind 10^3 – 10^4 more antibody molecules than do L-929 cells (Fig. 6). Second, preliminary experiments have demonstrated that our antibodies specifically immunoprecipitate a biosynthetically labeled protein from Vero cells (Rönnberg and Middlebrook, unpublished observations). Both these observations are inconsistent with the 'inhibitor' properties described by MEKADA *et al.* (1988) and suggest our monoclonal antibodies are against something else.

We believe that there are two likely explanations for the properties exhibited by the monoclonal antibodies. First, the antibodies are directed to an epitope representing a nonproteinaceous component on the receptor that is found at many other sites on the cell surface. Candidates could be sugars or more complex carbohydrates. The monoclonal antibodies are specific for this epitope, but it is an epitope shared by many other structures on the cell surface. Thus binding to the diphtheria toxin receptor, alone, cannot be measured above the background of specific binding to all those epitope sites on the cell. This hypothesis provides an explanation for the experiments (Fig. 7) where toxin, but not monoclonal antibody, binding was removed by treatment with trypsin or phospholipase C. If antibody binding to the diphtheria toxin receptor represents only 1% of the total antibody binding, it would be very difficult to detect that loss. Nevertheless, this hypothesis holds that antibody binds directly to a component of the receptor and should still be useful as an immunoprecipitating agent. We tested a few sugars, including Gal, Glu, Man, sucrose, lactose, *N*-acetyl-D-glucosamine, and D-galactose-amine, as competitors of antibody-cell binding, but obtained negative results (data not shown). However, this list is far from exhaustive and did not include more complex carbohydrates. The second possibility is that the monoclonal antibodies bind to a site adjacent to the receptor

and sterically, or by other means, indirectly block binding of the toxin to its receptor. In this case, the antibodies would not be useful as immunoreagents. Immunoprecipitation experiments now underway should allow us to distinguish between these two possibilities.

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